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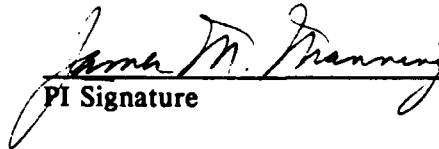
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## TABLE OF CONTENTS

	PAGE
Cover page	1
Report Documentation Page (DD Form 1473 and Abstract)	2
Foreword	3
Table of Contents	3
Introduction and Background	4-6
Experimental Methods	7-11
Results and Discussion	12-23
References	24-25

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## INTRODUCTION AND BACKGROUND

Our objective is to obtain a useful blood substitute with normal adult HbA as the starting material. In order for such a derivative to be useful it should have a low oxygen affinity to that oxygen can be readily released to the tissues. The experimental procedures should be as uncomplicated as possible and the materials should not be unduly expensive. To this end we have been studying the carboxymethylation of the N-terminal amino groups of HbA. The initial studies on this derivative showed that it had a low oxygen affinity, a desirable property for a blood substitute (1). An earlier annual report under this contract described the preparation of this derivative by a relatively simple procedure. The conclusions made from these studies will be briefly summarized here for purposes of continuity. Studies with other types of agents will be described herein.

The second objective of the studies is to cross-link this carboxymethylated Hb so that it will be retained longer in the circulation and yet maintain a significant degree of Hb function. For this purpose we are investigating the use of several types of crosslinking agents so that we might find the one with the most desirable properties (2,3). In this annual report, we focus on the reagents studied in the past year.

**Choice of the Carboxymethyl Group for a Hemoglobin-Based Blood Substitute** - For many years it has been known that small inorganic anions such as chloride lower the oxygen affinity of hemoglobin (4). This effect could be either a general, non-specific one due to increased ionic strength or due to the specific binding of chloride at certain sites on the hemoglobin molecule. The latter view has received experimental support from several laboratories (5-8). Earlier studies in our laboratory on hemoglobin specifically carbamylated at certain sites and on the mutant hemoglobin Providence indicated that two sites, i.e., Val-1( $\alpha$ ) and Lys-82( $\beta$ ) were major sites that accounted for 80% of the *oxygen-linked binding* of chloride to hemoglobin (9).

Studies on hemoglobin-based blood substitutes have been aimed at achieving a lowered oxygen affinity of hemoglobin to mimic that of hemoglobin within the intact red cell where the

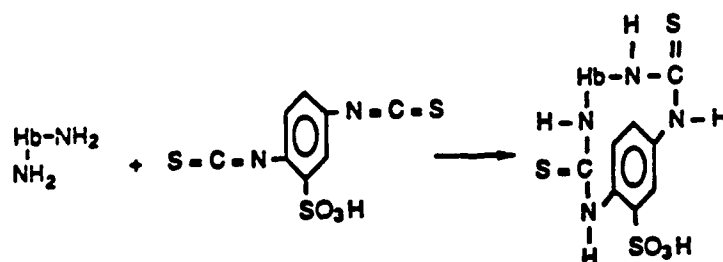
release of oxygen from hemoglobin is facilitated by its interaction with 2,3-DPG, protons, and CO<sub>2</sub> (10-12). Even though a lowered oxygen affinity for isolated hemoglobin can be achieved by addition of chloride, it is not possible to attain a *sustained* low oxygen affinity in this manner since the binding of this anion is reversible. The concept of preparing a covalent adduct of hemoglobin to mimic the effect of chloride is one that we have considered earlier (1,13,14). For this purpose, we have investigated the carboxymethylation of hemoglobin at specific sites to achieve a permanently anchored covalent anion (Hb-NH-CH<sub>2</sub>-COO<sup>-</sup>). The carboxymethyl group was chosen because it is a covalent anionic moiety of small size which we expected would not interfere drastically with any of the essential functions of hemoglobin.

The concept of neutralizing the positive charge in the DPG cleft to achieve a lower oxygen affinity has received support from studies in our laboratory and in others (8,9). Thus, the introduction of a carboxymethyl group at the terminus of the  $\beta$ -chain provides an additional negative charge in the DPG cleft and the oxygen affinity is thus lowered (13). This carboxymethyl group also mimicks the hemoglobin-CO<sub>2</sub> adduct (HbNH-COO<sup>-</sup>) (the reversible carbamino adduct) since under physiological conditions the binding of CO<sub>2</sub> to hemoglobin also lowers the oxygen affinity (11). Thus, two of the modulators of hemoglobin that lead to a lower oxygen affinity, i.e., chloride and CO<sub>2</sub> are not stably bound to hemoglobin. However, the carboxymethyl derivative is a stable covalent adduct that can be considered as a mimic for *both* of these structures.

**Crosslinking of Hemoglobin A** - In the past year we have sought a better crosslinking agent than glycoaldehyde, which we have investigated previously (2,3). Our results with glycoaldehyde indicated that some very high molecular weight polymerized hemoglobin was formed. Although it was possible to control the experimental conditions to a certain extent to achieve an enriched population of crosslinked species of a particular molecular weight, it did not seem possible to achieve the degree of control necessary to obtain a limited number of defined products with this crosslinking agent. Therefore, we screened a variety of different types of crosslinking agents including the diisothiocyanato types of crosslinkers - one with a

sulfonic acid (DIBS) moiety and the other with a benzoic acid (DIBA) moiety. These compounds are commercially available and are fairly pure as judged by elemental analysis performed in our lab.

An incentive for studying these crosslinkers in terms of generating a lower oxygen affinity is that they had the possibility of introducing a new negative charge (sulfonic acid or benzoic acid) into some region of the protein to produce a lower oxygen affinity by analogy to the carboxymethyl group at the  $\text{NH}_2$ -terminus of each chain, as described above. For several months we compared these two agents (DIBS and DIBA) and we found that both types of reaction were quite similar since SDS-gel electrophoresis showed predominantly a 32,000 molecular type of crosslinking and very little 48,000 or higher observed with glycoaldehyde. Therefore, the initial studies showed there was not extensive crosslinking to form large polymers



of hemoglobin. We elected to pursue in depth the chemistry of the crosslinking reaction with 2,5-diisothiocyantobenzene sulfonate (DIBS) (Eq. 1).

In the past year, we have combined our efforts with those of investigators at the Division of Blood Research, Letterman Army Institute of Research. Our current findings on this crosslinking agent, DIBS are described below.

## **Experimental Methods**

**Hemoglobins Preparations** - We have used hemoglobin prepared from a lysate of normal human erythrocytes by procedures we have used previously (1). We use normal adult hemoglobin. A mixed bed resin is used as an early step in the purification. For larger scale preparative studies we obtain units of outdated blood from the New York Blood Center.

**Crosslinking of Hemoglobin with Diisothiocyanato Benzene Sulfonic Acid (DIBS) and Diisothiocyanato Benzoic Acid (DIBA)** - We have studied a variety of experimental conditions such as ratio of crosslinker to hemoglobin, pH, incubation time, presence and absence of terminator after the reaction to optimize the crosslinking reaction in terms of a high degree of reaction to produce a limited number of products. The optimum conditions that we found are a 50:10:1 ratio of IHP:crosslinker:hemoglobin, pH of 7.2, and an incubation time of 15 min. Sodium triphosphate, an inexpensive compound, performs the same function as IHP. The reaction is then terminated by addition of a 30-fold excess (over DIBS) of Gly-Gly. Our earlier studies with the related, monofunctional sodium cyanate (7) showed that Gly-Gly is an efficient terminator. The yield of crosslinked products is 75-80% and the amount of met hemoglobin formed is less than 5%.

**Assay for DIBS** - The elemental analysis for the commercially available DIBS gives fairly good results for carbon, hydrogen, and nitrogen, as determined by the university microanalyst. The amount of DIBS bound to Hb (see below) can be determined by its absorbance at 295 nm.

**Estimation of Cross-Linking Between Hemoglobin Subunits** - For estimation of the amount of cross-linking between hemoglobin subunits, the dialyzed samples were subjected to SDS electrophoresis by the procedure of Laemmli (17) in 14% cross-linked gels. The amount of protein loaded onto each gel is in the range of 5-10  $\mu$ g. After the gel was stained with Coomassie Blue it is destained in 30% methanol and 5% acetic acid and the amount of each cross-linked subunit was estimated by densitometry on a Gilford Model 2520 instrument equipped with a Shimadzu integrator.

**Determination of Oxygen Equilibrium Curves of Hemoglobin Derivatives** - We have investigated a number of such properties of Cm-Hb, DIBS cross-linked Hb, and cross-linked CM-Hb. Some of the parameters are the oxygen equilibrium curves and in some instances the effect of the binding of modulators such as chloride and  $\text{CO}_2$  on the oxygen equilibrium curve. For selected cases we have also investigated the alkaline Bohr effect of some of these carboxymethylated and cross-linked derivatives using the procedures we have employed in the past (13). For the oxygen equilibrium curves we have used the Hem-O-Scan instrument which we have used in this laboratory (13,14). Recently, the Hemox instrument has also been used. We have now completely standardized the newer Hemox instrument so that we are confident of the data that it gives. The results obtained with this instrument compare favorably with those found on the Hem-O-Scan instrument (on the same sample) with which we have more experience.

The alkaline Bohr effect is measured by the proton release method or by the pH dependence of the oxygen affinity procedure; we have used both of these procedures previously. The oxygen equilibrium curves will be measured in the presence of various gas mixtures usually containing 25% oxygen and balance nitrogen either with or without  $\text{CO}_2$ . From the oxygen equilibrium curves the Hill coefficient,  $n$ , is calculated.

We have studied the effect of  $\text{CO}_2$  and chloride on several of the derivatives that we have prepared since any potential blood substitute will be exposed to these effectors present in plasma. The oxygen affinity may be lowered to a greater extent if the blood substitute is responsive to these effectors. It is clear from our results that the *deoxyhemoglobin* carboxymethylated and crosslinked by glycolaldehyde is responsive to both chloride and  $\text{CO}_2$  (16). Preliminary studies at one concentration of chloride have indicated that DIBS-crosslinked hemoglobin is partially responsive to chloride (see Table below). The oxygen equilibrium curve is performed in the presence of various gas mixtures usually containing 25% oxygen and balance nitrogen either with or without  $\text{CO}_2$ . From the oxygen equilibrium curves the Hill coefficient,  $n$ , is calculated. However, in view of the low oxygen affinity of some of these derivatives, it is

necessary to ensure that the sample is *fully* oxygenated. Therefore, in some cases we oxygenate the sample with a mixture of 50% O<sub>2</sub>, 50% N<sub>2</sub> that we can have specially prepared by the Alpha Gas Company in the tanks that can be attached to the Hem-O-Scan instrument.

The hemoglobin derivatives were dialyzed against 50 M Bis-Tris-acetate buffer, pH 7.5. Prior to determination of the oxygen equilibrium curve at 37°C, each sample is converted from the CO form to the oxy form as described previously (18) and then concentrated to about 0.5 mM in an Amicon Centricon 10 Microconcentrator or in an Amicon stirred cell with a YM10 membrane. The P<sub>50</sub> values were determined directly from the graphs of the Hem-O-Scan or Hemox as the oxygen tension corresponding to 50% of the maximal saturation attained (precision:  $\pm 1$  mm Hg). The Hill coefficients are estimated from the logarithmic values of the fractional saturation from 40-75% when plotted against the logarithmic values of the oxygen tension. The slope of this line gives the n value. In some experiments the effects of chloride and CO<sub>2</sub> on the hemoglobin derivatives are determined. These modulators were added to a concentration of either 0.1 or 0.2 M or in the appropriate tanks for the Hem-O-Scan containing O<sub>2</sub>/N<sub>2</sub> mixture and 5% CO<sub>2</sub> (for the latter studies the buffer strength was increased to 0.1 M Bis-Tris-acetate, pH 7.5).

**Mass Spectrometer Analysis for Protein-Bound DIBS** - Pendant DIBS moieties not crosslinked might be overlooked by the procedures of SDS gel electrophoresis. In addition, if a DIBS moiety occurs between 2 lysines in the *same* chain (i.e., such a subunit would not crosslinked) it might be missed unless a deliberate effort is made to detect it directly. Such studies have been initiated in the past year. The  $\alpha$ - and  $\beta$ -chain were separated by HPLC method described in (29). The uncrosslinked (i.e., 16 K) chain was collected and subjected to the mass spec procedure. A very sensitive procedure has been developed by Dr. Brian Chait at this institution that could readily be used to measure such moieties. During the past few months, efforts have been centered on preparing a 128,000 molecular weight crosslinked derivative.

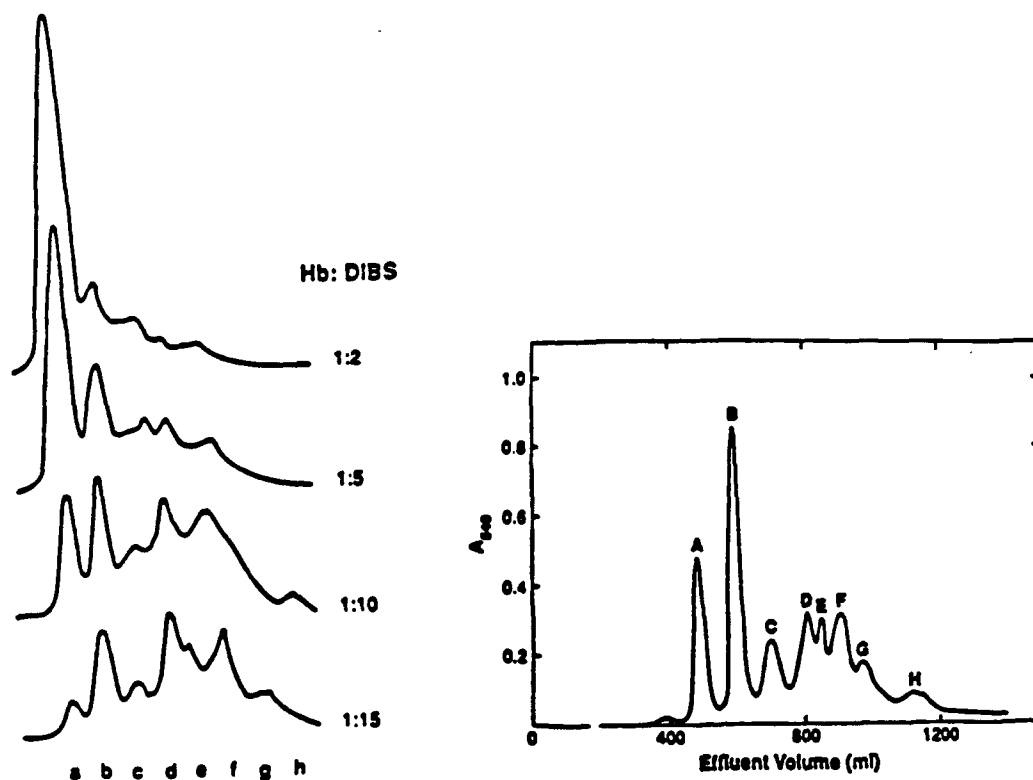
We start with Component B, a defined 64,000 molecular weight species from the DIBS-crosslinked hemoglobin. This component (at a high concentration to promote inter-tetrameric crosslinking) is then crosslinked with one of the imido esters in order to obtain a 128,000 molecular weight product. These compounds may act at a different site on hemoglobin as DIBS to make the second crosslinking possible. We have performed preliminary studies with some of the bifunctional imidates. Concentrations of 25 mM of DMA or DMS with 50  $\mu$ M hemoglobin solution crosslink hemoglobin quite efficiently. DMS is more potent than DMA. In theory, crosslinking could occur both within or between tetramers of Component B. These can be separated as described below.

The molecular weight of the products are ascertained by gel filtration on Sephadex G-100. We have already standardized a large column of Sephadex to separate 64,000 molecular weight from 128,000 molecular weight or greater species. The samples are analyzed by SDS-gel electrophoresis and gel filtration in the presence of 1 M  $MgCl_2$  to determine the degree of  $\alpha\beta$  dissociation, which is also the primary mode of dissociation under physiological conditions.

**Stability of Crosslinked Carboxymethylated Hemoglobin** - In order for a successful hemoglobin based blood substitute to be useful it must remain stable to various conditions of storage and not be oxidized to met hemoglobin. We have incubated sterile solutions (sterilized by filtration) of crosslinked or Cm-crosslinked hemoglobin at various temperatures (4°C, 0°C, -20°C, -80°C) and then determined by spectral means the amount of met hemoglobin formation (over a period of weeks). Preliminary studies indicate that Cm-hemoglobin (oxygenated) is less prone to oxidation than oxyhemoglobin at 4°C. Preliminary studies on DIBS cross-linked Component B also show that the cross-link is stable for months in sterile aqueous solution at room temperature.

**Studies on Plasma Retention Times** - One of the important questions that was answered in the past year is whether a crosslinked, carboxyalkylated hemoglobin derivative has a retention time *in vivo* compatible with its use as a blood substitute. We have prepared and purified well-defined hemoglobin derivatives for testing and sent the samples to Dr. John Hess at the Division

of Blood Research at the Letterman Army Institute of Research in San Francisco. Dr. Hess has determined the plasma retention time of uncrosslinked Cm-hemoglobin (Hb) with that of DIBS-crosslinked Hb by infusing six rats with a solution (about 10 ml) of the hemoglobin derivative at a concentration of about 13 gm/dL and six other rats with a concentration of about 7 gm/dL. The disappearance of the derivative from the plasma was then monitored by determination of the plasma hemoglobin concentration as a function of time. The kinetics of disappearance are then plotted and the half-life was calculated from the first-order plot. These results have been published (29).

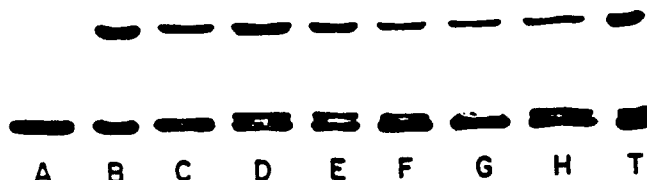


**Fig. 1 (left)** - Effect of Various Ratios of DIBS to Hemoglobin on the Degree of Crosslinking. The first component, a, is unmodified hemoglobin.

**Fig. 2 (right)** - Ion Exchange Chromatography of Hemoglobin Crosslinked by DIBS on DEAE-cellulose. Deoxyhemoglobin A (200  $\mu$ M) was treated with a 10-fold molar excess of DIBS and a 50-fold molar excess of IHP. The details are in the enclosed manuscript. The letters correspond to the peak tubes of each component that were analyzed for the extent of crosslinking in Fig. 3.

## Results

**Separation of Products** - We purify the DIBS crosslinked components by chromatography (Fig. 2). Each of the components is crosslinked (Fig. 3) (35-50% of 32,000 molecular weight band and no higher molecular weight bands are observed for any of the components. The amount of crosslinker on each component can be estimated since the DIBS crosslinker has an absorption maximum around 295 nm. Even though it has a low extinction coefficient we can get an approximation of how much DIBS is present in each component. The amount of DIBS moieties per hemoglobin tetramer varies from 1 for Component B up to about 3-5 for the latter components (F, G, H) in Fig. 2.



**Fig. 3** - SDS Electrophoresis of the Crosslinked Hemoglobin Components. The peak tubes of the Components designated A through H in Fig. 2 were subjected to SDS gel electrophoresis. The sample designated "T" refers to the total sample, i.e. before fractionation on DE-52. The lower band had a molecular weight of 16,000 and the upper band had a molecular weight of 32,000.

Component B constitutes the major product; the absolute amount depends upon the concentration of effectors such as chloride and DPG in the reaction mixture (see below). For example, the percentage of some of the components, i.e. B and F appear to be inversely related to one another and can range from 20-50% of the total depending on whether or not an effector such as IHP or chloride is present during the crosslinking reaction with DIBS.

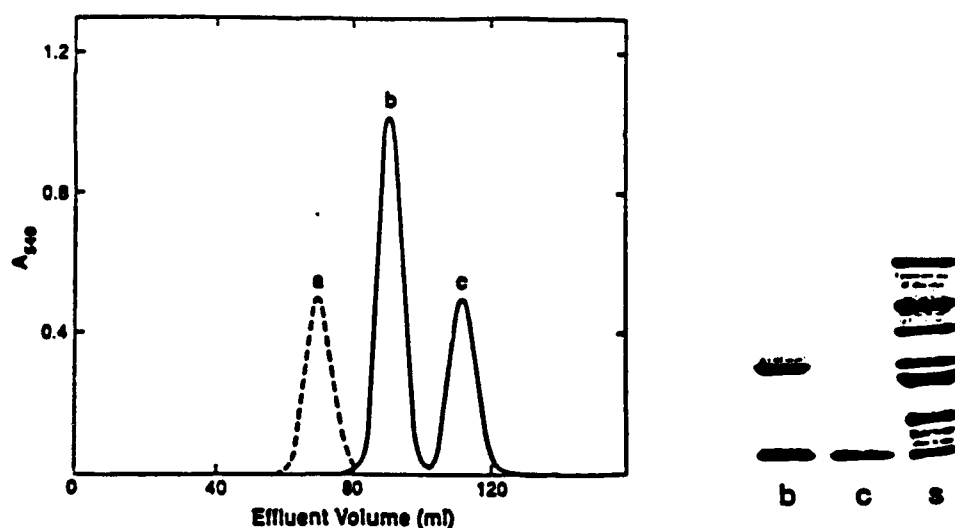
**Termination of Crosslinking** - In studies with the crosslinkers DIBS and glycoaldehyde (2) in which samples had been dialyzed extensively it has become apparent that some crosslinking continued to take place slowly. This might be due to the presence of pendant residues of crosslinker bound monofunctionally and therefore non-dialyzable. Studies in our lab and at Letterman by Drs. Marini and Currell have shown that this process continues slowly even at a reduced temperature (4°C). We have been able to prevent this latter type crosslinking and to control the products of the reaction by addition of various terminators at the end of incubation. Gly-Gly is the one most studied and found to be effective. Earlier studies in our lab showed that isonicotinic acid hydrazide (INH) is also an effective terminator but we prefer Gly-Gly since the presence of Gly can sometimes provide a useful marker than can be measured by amino acid analysis. Therefore, in our experience with crosslinking agents, we routinely use a terminator, such as an amine, to react with the excess crosslinking agent (either unreacted or present on the protein as a monofunctional entity) to prevent further uncontrolled, undesired crosslinking. This approach is also used by other investigators in this field, i.e. those who use 3,5-bis(dibromosalicylfumarate) as a crosslinker terminate the reaction by the addition of glycine (21). We prefer Gly-Gly because its pKa of its amine is lower and, therefore, it should be more efficient.

**Gel Filtration of Crosslinked Hemoglobin in the Presence or Absence of MgCl<sub>2</sub>** - The purpose of these studies was to establish the true molecular weight of the crosslinked derivatives (no MgCl<sub>2</sub>) and to learn something about the nature of the crosslink, i.e.,  $\alpha$ - $\alpha$ ,  $\beta$ - $\beta$  or  $\alpha$ - $\beta$  (with MgCl<sub>2</sub>). When the entire reaction mixture of DIBS crosslinked hemoglobin was placed on a column of Sephadex G-100 in the presence of 1 M magnesium chloride, a procedure by which hemoglobin dissociates into  $\alpha\beta$ -dimers, only two components were found, species b and c (Fig. 4).

Species b is crosslinked to the extent of 50% as determined by SDS gel electrophoresis (lane b of Fig. 5). Species c is not crosslinked (lane c of Fig. 5) and therefore it probably is derived from unmodified hemoglobin in the unfractionated reaction mixture (i.e., Component

A) and from subunits that are not crosslinked in any of the other components. Species a, which we found when the crosslinking agent was glycolaldehyde (3), is not present when DIBS is the crosslinking agent. The polymerized hemoglobin formed by potent agents, such as glutaraldehyde, would also elute in this position, i.e., extensive crosslinking between and within tetramers.

Gel filtration of the entire reaction mixture on Sephadex G-100 (sample unfractionated on DE-52) in the absence of magnesium chloride indicated that greater than 99% of the material was of molecular weight 64,000 and no 128,000 molecular weight or higher material was detected. Consistent with the conclusion that DIBS leads to crosslinking within a tetramer are the results on the effect of hemoglobin concentration on the crosslinking reaction. When two



**Fig. 4 (left)** - Gel Filtration of Crosslinked Hemoglobin in 1 M  $MgCl_2$ . The hemoglobin crosslinked with a 1:10:50 ratio of Hb:DIBS:IHP was used. Species a, designated by the dashed lines, was not present in this sample. Only Species b and c were present.

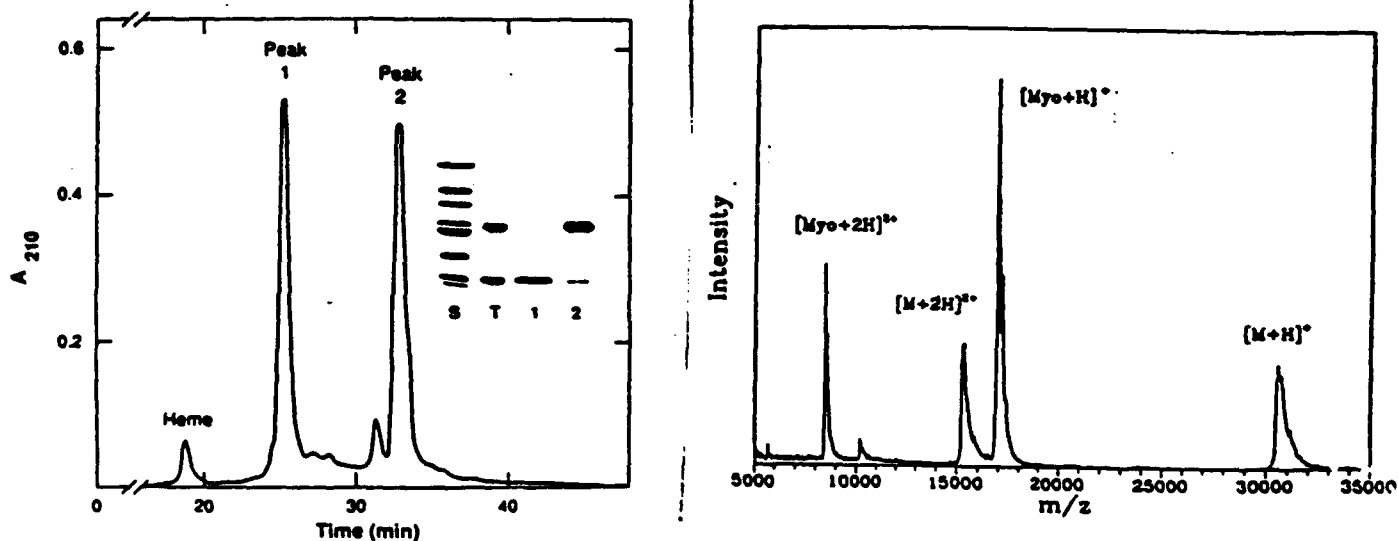
**Fig. 5 (right)** - SDS Gel Electrophoresis of Gel-Filtered Crosslinked Hemoglobin. Lane b contains sample b of Fig. 4. Lane c contains sample c of Fig. 4. Lane s contains a standard protein mixture of molecular weights 14,200, 20,100, 24,000, 29,000, 36,000, 45,000, 66,000 (bottom to top).

concentrations of hemoglobin (200  $\mu$ M and 350  $\mu$ M) were crosslinked with DIBS no significant dependence was observed on the pattern of crosslinking. This is the expected result if the crosslinking occurs within tetramers. On the other hand, crosslinking between tetramers would be expected to show a dependence on hemoglobin concentration, as we found previously with the crosslinking agent glycolaldehyde, as reported in previous progress reports.

**Partial Characterization of Component B of DIBS Crosslinked Hemoglobin -**  
Component B was the easiest to purify based on its elution position shown in Fig. 2. Therefore, it was possible to scale-up the procedure to obtain 2.5 gm for determination of its plasma retention time (described below) in collaboration with Dr. John Hess and his colleagues at Letterman Army Institute of Research. It was also possible to characterize this component to some extent since it could be purified easily. For example, Component B has one DIBS moiety per tetramer (as calculated from its  $A_{295}/A_{540}$ ) indicating there is one crosslink between two of the four subunits per tetramer, a result consistent with the finding, by SDS-gel electrophoresis, that 50% of the subunits were crosslinked. Gel filtration in the presence of 1 M magnesium chloride indicated that its molecular weight under these conditions was 64,000 (analogous to b in Fig. 4). This result suggests that the crosslink is between like subunits, either  $\alpha$ - $\alpha$  or  $\beta$ - $\beta$  since an  $\alpha\beta$ -crosslink would have permitted dissociation into the 32,000 molecular weight species, which was not found. Gel filtration in the *absence* of  $MgCl_2$  indicated that the molecular weight of Component B in its native state is 64,000.

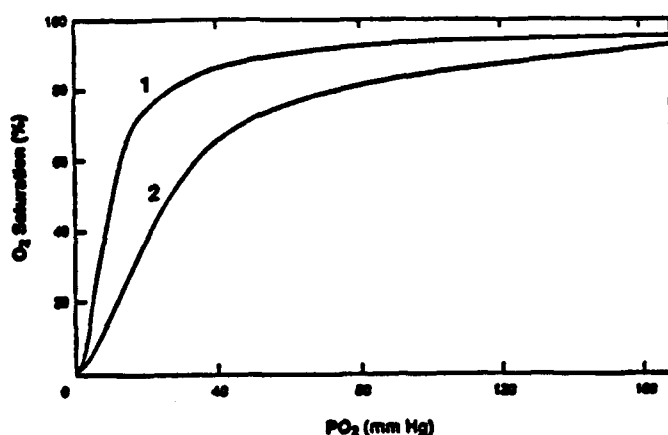
Some experiments on the identification of the crosslinked chains have been performed. Thus, when the p-hydroxymercurobenzoate procedure to separate the chains of human hemoglobin is applied to Component B, there are no free  $\alpha$ -chains found but a large peak is found in a different elution position. Furthermore, the HPLC procedure of Shelton et al. on a C-18 column with a 0.1% TFA/acetonitrile gradient has been used to separate the crosslinked material from the uncrosslinked chains and these samples have been subjected to amino acid analysis as described below.

The HPLC profile of Component B is shown in Fig. 6. SDS gel electrophoresis of the two components separated by HPLC according to the procedure of Shelton et al. show that one of them is 16,000 molecular weight (Peak 1) and the other is 32,000 molecular weight (Peak 2). The amino acid analysis of each component (Table I) clearly shows that Peak 1 is the  $\beta$ -chain and Peak 2 is the  $\alpha$ -chain of hemoglobin. Therefore, the single crosslink in Component B is located between the  $\alpha$ -chains. This is confirmed by analysis on the mass spectrometer (Fig. 6A).



**Fig. 6 (left)** - HPLC Separation of Component B. The first peak is heme. The protein in Peak 1 had a molecular weight of 16,000. The protein in Peak 2 had a molecular weight of 32,000 and represents the crosslinked subunits.

**Fig. 6A (right)** - Mass Spectral Analysis of Component B.



**Figs. 7** - Oxygen Equilibrium Curves of Crosslinked Hemoglobin. Line 1 is the oxygen equilibrium curve of hemoglobin crosslinked by DIBS in the oxygenated state. Line 2 is the oxygen equilibrium curve of hemoglobin crosslinked with DIBS in the deoxygenated state in the presence of IHP. The determinations were performed on the unfractionated sample at 37°C on a Hem-O-Scam at a hemoglobin concentration of 0.5-0.7 mM in tetramer in 50 mM Bis-Tris, pH 7.5 (no chloride).

**Oxygen Equilibrium Properties of Crosslinked Hemoglobin** - When deoxy hemoglobin was crosslinked by DIBS in the presence of IHP (inositol hexaphosphate) there is a very significant effect on the oxygen affinity as shown in Fig. 7. IHP is known to shift the allosteric equilibrium towards the deoxy conformation of hemoglobin. The  $P_{50}$  is decreased nearly 3-fold to 28 mm Hg with partial retention of cooperativity ( $n = 1.7$ ). This result is for the entire unfractionated mixture. The control hemoglobin had a  $P_{50}$  of 10 mm Hg. The crosslinking of hemoglobin A in the liganded state led to a slight increase in the oxygen affinity and a somewhat lower Hill coefficient (Table II). The effect of IHP on the crosslinking of *liganded* hemoglobin is also to shift the  $P_{50}$  slightly to the right to 19 mm Hg (Table I), when IHP is added to hemoglobin prior to the crosslinker. In the presence of IHP there is about a 7-fold decrease in the  $P_{50}$  of unmodified uncrosslinked hemoglobin (Table II).

The oxygen affinity of isolated Component B (Fig. 2) is not reduced ( $P_{50} = 9$  mm Hg,  $n = 2.2$ ). Therefore, the major contribution to the lower oxygen affinity of the unfractionated

**Table I - Identification of DIBS Crosslinked  
Chains of Component B by Amino Acid Analysis<sup>a</sup>**

<u>Amino Acid</u>	<u>Theoretical</u>		<u>Found</u>	
	<u><math>\beta</math>-Chain</u>	<u><math>\alpha</math>-Chain</u>	<u>Peak 1</u>	<u>Peak 2</u>
Lys	11	11	10.8	10.9
His	9	10	9.3	9.9
Arg	3	3	2.9	2.8
Asp	13	12	13.0	12.2
Thr	7	9	6.8	8.3
Ser	5	11	5.3	10.1
Gly	<i>11</i>	<b>5</b>	<i>11.8</i>	<b>5.6</b>
Pro	7	7	8.2	8.3
Gly	<i>13</i>	<b>7</b>	<i>12.8</i>	<b>7.2</b>
Ala	<i>15</i>	<b>21</b>	<i>15.1</i>	<b>20.4</b>
Val	<i>18</i>	<b>13</b>	<i>16.9</i>	<b>12.5</b>
Ile	0	0	0	0
Leu	18	18	18	18
Phe	8	7	8.1	7.2

<sup>a</sup> The amino acids shown in bold face ( $\alpha$ -chain) or in italics ( $\beta$ -chain) are those for which significant differences exist between the  $\alpha$ - and  $\beta$ -chains. The values for Cys, Met, Tyr, and Trp are not included because these amino acids are either partially or completely destroyed during acid hydrolysis; the special conditions for hydrolysis or analysis to protect these amino acids were not needed for purposes of identification of the chain. Since the recovery of the amino acids listed is nearly complete, it appears likely that the DIBS crosslink is completely hydrolyzed upon acid hydrolysis in 6 N HCl with regeneration of the amino acid side chain to which it was attached.

**Table II - Effect of Allosteric Effectors on the  
Oxygen Affinity of Hemoglobin Crosslinked by DIBS**

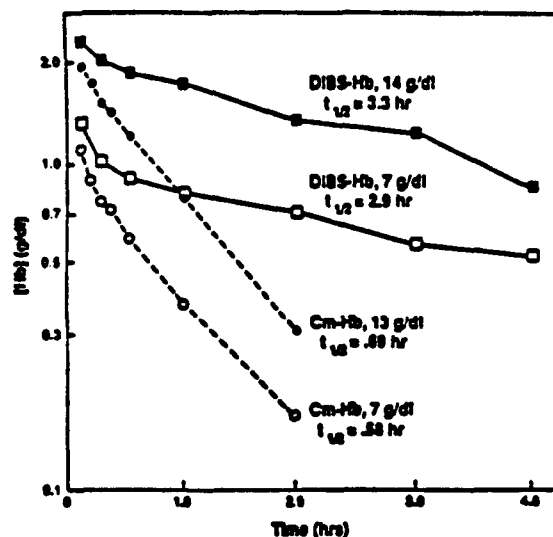
<u>Sample</u>	<u>Addition</u>	<u>P<sub>50</sub></u> mm Hg	<u>n</u>
OxyHb	--	12	2.2
	0.1 M NaCl	16	2.1
	5 mM IHP	80	2.6 <sup>c</sup>
CmHb <sup>a</sup>	-	28	2.0
OxyHb + DIBS	--	11 <sup>b</sup>	1.5
	--	10	1.4
	0.1 M NaCl	12	1.8
	5 mM IHP	13	1.7
	--	15 <sup>b</sup>	1.6
OxyHb + IHP + DIBS	--	19	1.5
	0.1 M NaCl	17	1.5
	5 mM IHP	17	1.5
	--	10 <sup>b</sup>	1.8
	--	10	1.3
DeoxyHb + DIBS	0.1 M NaCl	11	1.4
	5 mM IHP	11	1.6
	--	24 <sup>b</sup>	1.7
	--	28	1.6
DeoxyHb + IHP + DIBS	0.1 M NaCl	25	1.8
	5 mM IHP	23	1.7
	--	24 <sup>b</sup>	1.7
	--	28	1.6

The crosslinking experiments were performed in the presence of IHP as described in the text. The P<sub>50</sub> values were determined on the unfractionated reaction mixture at a hemoglobin concentration between 0.5 and 0.7 mM. When duplicate determinations were performed, at pH 7.5 and 37°C on a Hem-O-Scan instrument, the precision was  $\pm 1$  mm Hg. The extent of Met Hb formation averaged less than 5%.

<sup>a</sup> Hemoglobin A was carboxymethylated as described in reference #7 and had been stored at -80°C for 12 months prior to this measurement. The P<sub>50</sub> of freshly prepared CmHb was 28 mm Hg.

<sup>b</sup> After the crosslinking reaction with DIBS, the glycylglycine terminator was not added. In all other studies, the crosslinking was stopped by the addition of a 30-fold molar excess of glycylglycine as described in the text.

<sup>c</sup> The Hill coefficient (n) was calculated from the 40-70% range of the oxygen equilibrium curve.



**Fig. 8** - Plasma Retention Time of Cm-Hemoglobin and DIBS-Hemoglobin in Rats.

mixture of crosslinked products (Fig. 7) must be due to one or more of the other components (C-H), which are not yet completely separated. Preliminary experiments with a preparation enriched in Component F (but not yet pure) showed that its  $P_{50}$  in the range of 30-35 mm Hg. It is possible that some of the latter Components E and G also have low oxygen affinities. Subsequent studies, as described in the present application, will define the  $P_{50}$  of each of the purified components, the position of its crosslink, and the retention time of selected samples as described below.

**Plasma Retention Times of Uncrosslinked and of Crosslinked Hemoglobin A** - In collaboration with Dr. John Hess and Dr. Robert Winslow at the Letterman Army Institute of Research, the plasma retention time of several hemoglobin derivatives was determined in rats during the past year. First, the retention time of purified carboxymethylated hemoglobin (Cm-hemoglobin) 64,000 molecular weight, uncrosslinked,  $P_{50} = 28$  mm Hg was measured (Fig. 8). The data show that at two concentrations of infused hemoglobin, i.e., 13 gm/dL and 7 gm/dL, the retention time of the carboxymethylated hemoglobin was 35 min and 41 min, respectively. Thus, Cm-hemoglobin, which has a low oxygen affinity, is cleared from the circulation at nearly the same rate as unmodified native hemoglobin with a high oxygen affinity.

Dr. Hess and his colleagues then determined the retention time of Component B purified from the DIBS crosslinked products as described above. Its plasma retention time in rats was

found to be 2.9 and 3.3 hrs at the same two concentrations of hemoglobin used in the study with Cm-hemoglobin. Thus, this crosslinked derivative has a very significantly increased retention time, of the same order of magnitude of the retention time of the  $\alpha$ - $\alpha$  crosslinked hemoglobin after reaction of hemoglobin with bis(3,5-dibromosalicyl) fumarate, a low affinity derivative. We conclude from these results that the plasma retention time is simply a function of the crosslinking of the protein and is not dependent on the oxygen affinity of the hemoglobin derivative.

**Other Hemoglobin Cross-linkers** - The reaction of human hemoglobin A with methoxy polyethylene glycol (MPG) p-nitrophenyl ester takes place very smoothly. There is about 40-50% crosslinking of this particular MPG-hemoglobin. This MPG-hemoglobin derivative also behaves as one species on gel filtration in Sephadex G100. Its molecular weight is in the region of 128K. If the gel filtration is carried out in the presence of 1M magnesium chloride, there is no splitting of the derivative into individual  $\alpha$  or  $\beta$  subunits. When the MPG-hemoglobin is prepared under anaerobic conditions, the  $P_{50}$  is in the range of about 20mm of mercury with an n value of 1.7 (control unmodified hemoglobin  $P_{50}$  = 10mm of mercury, n=2.2). These studies will be continued.

**Other Carboxyalkylating Agents Under Study** - We have begun to investigate longer carboxyalkyl substituents on hemoglobin. Thus, the carboxypropyl derivative has been prepared by treating hemoglobin with succinic semi-aldehyde (SSA) in the presence of NaCNBH<sub>3</sub>. The reaction is fairly efficient with good yields obtained with a ratio of Hb: SSA:NaCNBH<sub>3</sub>. At the present time we have separated four major products by chromatography on CM-52. We have also prepared synthetic standards of SSA-valine and SSA-lysine (with <sup>3</sup>H-NaCNBH<sub>3</sub>) and the positions these have been placed on the amino acid analyzer. Each of the above components is being analyzed for the distribution of these and the one(s) with the highest SSA-Val/SSA-Lys ratios will be further tested for their  $P_{50}$  values.

## Discussion

The reductive carboxymethylation of hemoglobin does not adversely affect the native structure of the protein as shown by the functional studies and the X-ray diffraction results. The major effect in the functional properties of carboxymethylated hemoglobin is its lower oxygen affinity, which is a desirable property for a blood substitute. Reductive carboxymethylation is directed exclusively at the amino groups of hemoglobin since these are the only amino acid side chains that form Schiff base adducts with sodium glyoxylate that are reducible by sodium cyanoborohydride. The selective modification at the N-terminal amino groups of the  $\alpha$ - and  $\beta$ -chains of hemoglobin was achieved after testing a variety of conditions such as varying pH and ratios of reactants. This fact has been established by peptide mapping and by X-ray diffraction analysis.

The carboxymethyl moiety attached to hemoglobin is a small stable covalent adduct which is much more stable and hence easier to study than the carbamino adduct which forms a weak linkage with hemoglobin. Thus, the low oxygen affinity of the carboxymethyl derivative ( $\text{HbNHCH}_2\text{COOH}$ ) mimics that of the carbamino adduct ( $\text{HbNHCOOH}$ ). The X-ray diffraction maps of carboxymethyl hemoglobin and of carbamino hemoglobin are strikingly similar, not only with respect to the location of the adducts but, more importantly, to their interaction with other parts of the protein. It has been appreciated for some time that the  $\text{CO}_2$ -hemoglobin (carbamino) adduct has a low oxygen affinity but the molecular basis for this has been difficult to establish because of the lability of the carbamino adduct. The lower oxygen affinity of carboxymethyl Hb is apparently due in part to the fact that the carboxymethyl group on the N-terminus of the  $\beta$ -chain protrudes deep into the DPG cleft as observed by X-ray diffraction analysis. This interaction leads to a neutralization of positive charge in this cleft between the two  $\beta$ -chains and such an interaction is known to lead to a lower oxygen affinity. This effect has been previously observed in studies both with chemically modified (7,27,28) and mutant hemoglobin Providence A (8). Thus, the introduction of the covalent carboxymethyl group at this site mimics the effects of the binding of chloride, DPG, or  $\text{CO}_2$ , which are modulators that

neutralize positive charges within this site. Such neutralization of positive charges at this site stabilizes the deoxy or T structure of hemoglobin.

One of the concepts that has emerged from our studies and those from other labs is that of "freezing" of a given hemoglobin conformation as a function of its state of oxygenation during the crosslinking. Thus, the current studies with the crosslinking agent DIBS as well as the earlier studies with the crosslinker, glycolaldehyde, support this conclusion. This concept is developed more fully in the next section with examples from the work of other investigators. However, more examples are needed before a generalization can be made.

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